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CHAPTER 18

Approaches for the generation of new anti-cytomegalovirus agents: Identification of protein-protein interaction inhibitors and compounds against the HCMV IE2 protein

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Running Head: Assays to discover new anti-HCMV compounds

Human cytomegalovirus (HCMV) infection is responsible for severe, often even fatal, diseases in immunocompromised subjects and also represents the major cause of viral-associated congenital malformations in newborn children. The few drugs licensed for anti-HCMV therapy suffer from many drawbacks and none of them have been approved for the treatment of congenital infections. Furthermore, the emergence of drug-resistant viral strains represents a major concern for disease management. Thus, there is a strong need for new anti-HCMV drugs. Here we describe three different assays for the discovery of novel anti-HCMV compounds: two are *in vitro* assays, i.e., a fluorescence polarization (FP)-based assay and an enzyme-linked immunosorbent assay (ELISA) which are designed to search for compounds that act by disrupting the interactions between the HCMV DNA polymerase subunits, but in general can be employed to find inhibitors of any protein-protein interaction of interest; the third is a cell-based assay designed to identify inhibitors of the viral immediate-early 2 (IE2) protein activities.

Key words: antivirals, cell-based assay, DNA polymerase inhibitors, enzyme-linked immunosorbent assay, fluorescence polarization, human cytomegalovirus, IE2 protein, protein-protein interaction.

1. Introduction

Human cytomegalovirus (HCMV) infection is associated with severe morbidity and mortality in immunocompromised individuals, such as transplant recipients and AIDS patients, and is also the most frequent cause of viral-associated congenital defects in newborn children. The few drugs currently licensed for anti-HCMV therapy, most of which target the catalytic activity of the viral DNA polymerase, suffer from many drawbacks, including low potency, poor bioavailability, and long-term toxicity; furthermore, none of these drugs have been approved for the treatment of congenital infections. Additionally, due to the fact that most of the current anti-HCMV drugs share the same mechanism of action, the emergence of drug-resistant viral strains is becoming an increasing problem for disease management. Thus, there is a strong need for novel anti-HCMV drugs that are more potent, less toxic, and possibly act by a mechanism of action different from that of the currently available drugs.

The viral DNA polymerase represents a major target for anti-virals *(1)*, and new compounds that act by disrupting the interactions between the polymerase subunits rather than inhibiting the enzyme catalytic activity have been recently discovered, thus demonstrating the feasibility of alternative antiviral strategies *(2)*. In addition, the earliest events of the virus replication cycle, in particular the functions of the immediate-early 2 (IE2) protein, represent attractive targets for the development of novel antiviral compounds *(3)*. In fact, the multifunctional IE2 protein is crucial for regulation of viral early (E) gene expression and there is compelling evidence that it plays a direct role in the pathogenesis of HCMV infection by inducing a broad dysregulation of host gene expression. This leads to changes in host cell physiology and contributes to HCMV-induced cell cycle alterations, immunomodulation, and proinflammatory responses. Thus, molecules that inhibit IE2-dependent activities may be effective in blocking the virus-induced pathological phenomena at an early stage of

infection. Moreover, inhibitors of IE2 could be of particular importance for the treatment of patients who do not respond to currently available inhibitors of viral DNA replication.

In this chapter we will describe novel approaches to develop new compounds against HCMV. First, we will focus on two kinds of *in vitro* assays to identify compounds able to inhibit protein-protein interactions essential for HCMV replication. As an example, we will describe two different methods for identifying inhibitors of the interactions occurring between the two subunits of HCMV DNA polymerase, i.e., UL54 and UL44, which are essential for viral genome replication. However, these approaches can be applied to virtually every molecular interaction for which the interacting domains have been identified.

The first assay is based on measurement of fluorescence polarization (FP), which is a simple and rapid method to study molecular interactions and their inhibition. The basic principle of an FP-based assay is that a peptide or another molecule of small size (e.g., an oligodeoxynucleotide) labelled with a fluorophore rotates rapidly when free in solution. When the conjugated fluorophore is excited with plane polarized light, the emitted fluorescence is depolarized (i.e., in a plane different from the excitation light). When the rotation is slowed down due to an interaction with a molecule of greater size (e.g., a protein or another kind of ligand), the fluorophore tumbles slowly with respect to fluorescence life-time and hence the emitted light remains polarized. The presence of an inhibitor of the molecular interaction between the small, labelled molecule and the large ligand causes a depolarization that can be quantitatively detected by using an FP reader. The advantages of FP assays are that the detection limits are in the sub-nanomolar range, it is performed in solution, it does not require immobilization or washing steps, it is very rapid, and it permits quantitative measurements. Altogether, these features render an FP-based assay suitable for the screening of a large number of compounds, and thus it is very useful for high-throughput screenings (HTS). In addition, FP-based assays can be used for the identification of inhibitors not only of protein-protein interactions, but also of protein-nucleic acid

interactions (e.g., binding of transcription factors to specific promoters or other viral DNA or RNA sequences) *(4, 5)*. **Fig. 1** illustrates the principles of an FP-based assay to discover inhibitors of protein-protein interactions.

The second approach is based on the enzyme-linked immunosorbent assay (ELISA) and is recommended for the screening of a restricted number of compounds, for example for testing compounds identified from *in silico* screenings of small-molecule structures or for the validation of compounds discovered by HTS. However, this assay can be an alternative to the FP-based assay for those who do not possess a FP reader.

Finally, we will describe a cell-based assay designed to discover inhibitors of IE2 protein activities. This assay is based on new reporter cell lines derived from a stably transfected human cell line permissive to HCMV replication, in which the expression of the enhanced green fluorescent protein (EGFP) reporter gene is driven by the HCMV E *UL54* or *UL112/113* gene promoters that can betransactivated by the IE2 protein alone *(6, 7)*. The EGFP-expressing cell lines show a specific and inducible dose- and time-dependent EGFP response to either HCMV infection or constitutive IE2 expression that can be visually assessed by fluorescence microscopy or evaluated by automated fluorometry*(8)*. The reliability of these reporter cell lines for assessing virus-inhibitory effects in concentration- and time-dependent fashions after HCMV infection or IE2 transfection has been demonstrated by treatment with fomivirsen, an antisense oligodeoxynucleotide designed to block IE2 expression *(8, 9)*, and with WC5, a novel 6-aminoquinolone endowed with potent inhibitory activity for HCMV replication *(10)*. Thus, these EGFP-based cell lines can be exploited as a tool to screen antiviral compounds that specifically target IE2 expression and/or function.

2. Materials

2.1 Fluorescence polarization (FP) assay

1. Recombinant Glutathione S-Transferase (GST)-UL44ΔC290 fusion protein expressed from *Escherichia coli* BL21 (DE3) pLysS (Invitrogen) harbouring the appropriate plasmid and purified as previously described *(11)*.

2. Synthetic peptides corresponding to the 22 C-terminal residues (LPRRLHLEPAFLPYSVKAHECC) of HCMV UL54 protein, which have been described in *(12)*. Both Unlabelled peptide and peptide labelled at the N-terminus with the pentafluorofluorescein-derivative Oregon Green 514 (Molecular Probes) will be needed for these assays.

3. FP Buffer: 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol (DTT), 0.5 mM EDTA, 150 mM NaCl, 4% glycerol, and 100 µg/ml of bovine serum albumin (BSA).

4. Collection of small-molecule compounds to be tested in the FP assay, preferably resuspended in dimethyl sulfoxide (DMSO) at concentrations between 10 and 50 mM (*see* **Note 1**).

5. 384- or 96-well black plates.

6. Automated dispenser for 384- or 96-well plates (*see* **Note 2**).

7. FP reader (for example, Analyst Plate Reader, LJL Biosystems).

2.2 Enzyme-linked immunosorbent assay (ELISA)

1. ELISA microtiter plates. Microtiter plates with high protein-binding capacity are recommended (e.g., Immulon 1, Dynatech).

2. Recombinant HCMV UL54 and UL44 proteins expressed from baculovirus-infected *Sf*9 insect cells and purified as described *(12)*. The UL44 protein has an EEF epitope tag fused at the C terminus.

3. 2% BSA solution in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4).

4. Wash Buffer: 0.3% Tween 20 in PBS.

5. Synthetic peptide corresponding to the 22 C-terminal residues (LPRRLHLEPAFLPYSVKAHECC) of HCMV UL54 protein, which is a known inhibitor of UL54-UL44 interaction *(12)*, dissolved in 100 mM Tris-HCl pH 8.0 with 0.1% Tween 20.

6. Collection of small-molecule to be tested in ELISA, preferably resuspended in DMSO at concentrations between 10 and 50 mM (*see* **Note 1**).

7. Monoclonal Antibody (MAb) YL1/2 (Serotech Ltd), which recognizes an EEF epitope tag *(13)* inserted at the C terminus of UL44 protein.

8. Horseradish peroxydase (HRP)-conjugated anti-rat antibody.

9. Chromogenic substrate 2,2'-azino-bis(3-ethylbenzthiazinzoline-6-sulfonic acid (ABTS) in citrate phosphate buffer (pH 4.0) containing 0.01% hydrogen peroxide.

10. 3 N NaOH.

11. ELISA plate reader with absorbance filter at 405 nm (for example, Sunrise Reader, Tecan).

2.3 Cell-based assay

2.3.1. Cultivation of the reporter cells lines 2F7 and 1B4

1. U373-MG UL54-EGFP (clone 2F7) and U373-MG UL112/113-EGFP (clone 1B4) cell lines are derived from the astrocytoma/glioblastoma cell line U373-MG (*see* **Note 11**).

2. G418 stock solution (50 mg/ml).

3. Growth medium for 2F7 and 1B4 cells: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin sulphate, 750 µg/ml G418.

4. PBS.

5. 100 μg/ml trypsin and 2 mM EDTA in PBS.

2.3.2. Infection of the reporter cells lines 2F7 and 1B4

1. HCMV stocks: HCMV laboratory strainAD169 (ATCC, #VR-538) is propagated and titered in low-passage human embryonic lung fibroblasts (HELF) by standard protocols. HCMV clinical isolates are propagated in low-passage human umbilical vein endothelial cells (HUVEC) and titered by the indirect immunoperoxidase staining procedure on HELFs using a MAb reactive to the HCMV IE1 and IE2 proteins (*see* **Note 12**).

2. Maintenance medium for HCMV-infected 2F7 and 1B4 cells: DMEM supplemented with 5% FBS and 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate.

2.3.3. Quantification of EGFP expression in HCMV-infected 2F7 and 1B4 cells by fluorescence microscopy

1. Glass coverslips in 24-well plates.

2. PBS.

3. Fixing solution: 4% paraformaldehyde in PBS.

4. Mounting medium (e.g., Vectashield).

2.3.4. Quantification of EGFP expression HCMV-infected 2F7 and 1B4 cells by automated fluorometry

1. 24- or 96-well culture plates.

2. PBS.

3. NP-40 Lysis buffer: 50 mM Tris-HCl pH 7.8, 150 mM NaCl, 5 mM EDTA, 15 mM $MgCl_2$, 0.15% NP-40.

4. 96-well plate suitable for fluorescence measurement (e.g., Nunc #136101).

2.3.5. Validation of 2F7 and 1B4 reporter cell lines for selection of inhibitors of IE2 protein activity

1. Antiviral stock solutions: 1 mM fomivirsen (phosphorothioate oligodeoxynucleotide 5'-GCGTTTGCTCTTCTTCTTGCG-3', dissolved in10 mM Tris-HCl pH 8.0, 1 mM EDTA); 25 mM WC5 synthesized and dissolved in DMSO as described in *(10)*. Fomivirsen (ISIS 2922) is 21-base phosphorothioate oligodeoxynucleotide complementary to the IE2 mRNA *(9)* that has been approved for intraocular application in patients with HCMV retinitis. WC5 is a 6-aminoquinolone derivative that has been shown to exert an inhibitory activity on HCMV replication by interfering with the IE2-dependent transactivation of the early genes UL54 and UL112/113 *(10).*

3. Methods

3.1 Fluorescence polarization (FP) assay to search for inhibitors of HCMV DNA polymerase subunit interactions

1. Mix 2.5 µM GST-UL44ΔC290 and 3 nM of the Oregon Green labelled UL54 peptide in FP buffer and incubate on ice (*see* **Note 3**).

2. Dispense the appropriate volume of the reaction mixture containing the labelled peptide and GST-UL44ΔC290 protein into 384-well black plates (40 µl of reaction mixture) or into 96-well plates (160 µl of reaction mixture) (*see* **Note 4**).

3. Add potential small molecule inhibitors to the 96 or 384 well plates at a final concentration of 12.5 µg/ml, or the same volume of compound vehicle (e.g., DMSO; *see* **Note 7**), to exclude potential inhibitory effects due to compound vehicle. It is preferable to test each potential inhibitor in duplicate. As controls, include wells with the UL54-derived peptide and GST-UL44ΔC290 protein mixture with

no compound and also include wells with the UL54-derived peptide only (*see* **Note 5**). In addition, as a positive control for inhibition of the interaction between UL44 and UL54, add unlabelled UL54-derived peptide at a final concentration of 20 µM in duplicate wells.

4. Incubate at room temperature (RT) for 15 min.

5. Measure FP values from each well (expressed as millipolarization units) using an appropriate FP plate reader.

6. Typical results of an FP-based assay designed to search for inhibitors of the interaction between the two subunits, UL54 and UL44, of HCMV DNA polymerase are shown in **Fig. 2**.

3.2 Enzyme-linked immunosorbent assay (ELISA) to search for inhibitors of HCMV DNA polymerase subunit interactions

1. Use 0.2 µg/well of purified, baculovirus-expressed UL54 protein dissolved in PBS for the coating of ELISA microtiter plates and incubate at 37°C overnight.

2. Aspirate solution containing unbound protein and wash each well five times with 0.2 ml of Wash Buffer (*see* **Note 6**).

3. Block with 0.1 ml/well of 2% BSA in PBS at RT for 1 h.

4. Aspirate and wash as described in **Step 2**.

5. Add 0.5 µg/well of purified, baculovirus-expressed UL44 protein dissolved in PBS alone or mixed with test compounds (previously dissolved in DMSO) at the desired final concentration in duplicate wells (*see* **Notes 7** *and* **8**). As controls, include wells with the UL54 protein alone (i.e, do not add UL44 protein and inhibitors, but add the same volume of PBS; this is a control for antibody specificity), wells coated with the UL54 protein and incubated with UL44 protein mixed with the UL54-derived peptide (the UL54-derived peptide is a known inhibitor of UL54-UL44 interaction; thus, this is a positive control for inhibition), and wells coated with the UL54 protein and incubated with UL44 protein mixed

with compound vehicle (e.g., DMSO; *see* **Note 7**). The latter control is to exclude inhibitory effects due to compound vehicle.

6. Incubate the plates at 37°C for 1 h to allow protein-protein interaction.

7. Aspirate and wash as described in **Step 2**.

8. Add 50 µl/well of YL1/2 MAb diluted 1:50 in PBS containing 2% FBS and incubate at 37°C for 1 h in the dark.

9. Aspirate and wash as described in **Step 2**.

10. Add 50 µl/well of HRP-conjugated anti-rat antibody diluted 1:500 in PBS with 2% FBS and incubate at RT for 1 h.

11. Aspirate and wash as described in **Step 2**.

12. Add 100 µl/well of ABTS solution (prepared as described in section 2, **Materials**) and incubate at RT for 15 min in dark (*see* **Note 9**). Block the reaction by adding 50 µl/well of 3 N NaOH (*see* **Note 10**).

13. Measure absorbance at 405 nm using an appropriate ELISA reader.

14. Typical results of an ELISA-based assay designed to search for inhibitors of the interaction between the two subunits, UL54 and UL44, of HCMV DNA polymerase are shown in **Fig. 3**.

3.3 Cell-based assay

3.3.1. Cultivation of the reporter cells lines 2F7 and 1B4

1. For maintenance, 2F7 and 1B4 cells are routinely cultured in DMEM-10% FBS containing 750 µg/ml G418. Cells up to 60th passage can be used for HCMV quantification and antiviral assays.

2. Cells are subcultured by trypsinization with a solution of 100 µg/ml trypsin in PBS-2 mM EDTA solution to obtain new maintenance cultures using a split ratio of 1:3. It is recommended to subculture

the cells prior to reaching confluence.

3.3.2. Infection of the reporter cells lines 2F7 and 1B4

1. Seed 2F7 and 1B4 cells in 24- or 96-well plates at a density of 70×10^3 or 10×10^3 cells/well, respectively.

2. After 24 h, remove the growth medium by aspiration, and immediately add a small volume of viral inoculum (0.25 ml for 24 well plate or 0.05 ml for 96 well plate) that is prepared by diluting viral stocks solutions in maintenance medium for HCMV-infected cells to obtain the appropriate multiplicity of infection (MOI). Plates should be gently rocked to achieve even distribution of the viral inoculum.

3. Incubate infected cells for 2 h at 37°C in a humidified incubator with 5% CO_2 to allow virus adsorption.

4. Remove viral inoculum by aspiration and add an appropriate volume of DMEM with 5% FBS to each well and incubate in a CO_2 incubator at 37° C.

3.3.3. Quantification of EGFP expression in HCMV-infected 2F7 and 1B4 cells by fluorescence microscopy

1. Prepare 2F7 and 1B4 cells on glass coverslips in 24-well plates and infect cells with HCMV AD169 or HCMV clinical isolates as described in **Subheading 3.3.2.**

2. Prepare in advance the following solutions: precooled (on ice) PBS and fixing solution.

3. At the appropriate times post-infection (p.i.), remove medium from mock- and HCMV-infected cultures and gently wash cells twice with ice cold PBS (*see* **Note 13**).

4. Aspirate the residual PBS from cell cultures and place the plates on ice and add 0.25 ml of fixing solution to each well.

5. Incubate the plates at RT for 20 min and gently wash cells twice with ice cold PBS, then mount

coverslips with mounting medium.

6. Examine EGFP-expressing cells by fluorescence microscopy in combination with conventional phase contrast (e.g., Olympus Fluoview-IX70 inverted confocal laser scanning microscope). A 10× objective lens and a fixed data collection time of 0.2 s are used for the conventional assay.

7. Evaluate the numbers of EGFP expressing cells in captured images using an image processing software package (e.g., Image J).

8. Fig. 4 shows a typical result of a fluorescent microscopy evaluation of EGFP expression in 2F7 and 1B4 cells infected with HCMV AD169 for 72 and 96 h (*see* **Note 13**).

3.3.4. Quantification of EGFP expression in HCMV-infected 2F7 and 1B4 cells by automated fluorometry

1. Prepare 2F7 and 1B4 cells seeded in 24- or 96-well plates and infect cells with HCMV AD169 or HCMV clinical isolates as described in **Subheading 3.3.2.**

2. The day of the experiment, prepare precooled (on ice) PBS and NP-40 lysis buffer.

3. At the appropriate times p.i., remove medium from mock- and HCMV-infected cultures and gently wash cells twice with ice cold PBS.

4. Aspirate the residual PBS from cell cultures and place the plates on ice. Add 0.05 ml or 0.25 ml of NP-40 lysis buffer to each well of 96-well or 24-well plates, respectively.

5. Incubate at 4°C for 30 min. Carefully transfer the lysates to 0.5 ml labelled microcentrifugation tubes, and clarify by centrifugation at 12,500 rpm for 10 min at 4°C.

6. Transfer 0.05 ml of the supernatants to a 96-well plate suitable for fluorescence measurement.

7. Measure the EGFP fluorescence content in a multiwell fluorescence plate reader (e.g., Perkin Elmer Victor^3 1420 Multilabel Counter) with excitation and emission filters set at 485 and 530 nm, respectively.

8. Calculate the net EGFP intensity of infected cells by subtracting the fluorescence intensity of the mock-infected cells from that of HCMV-infected cells. Express the HCMV infectivity as fluorescence units (*see* **Note 14**).

3.3.5. Validation of 2F7 and 1B4 reporter cell lines for selection of inhibitors of IE2 protein activity

1. Prepare 2F7 and 1B4 cells seeded in 24- or 96-well plates as described in **Subheading 3.3.2.**

2. For cultures to be treated with fomivirsen, add different concentrations of the antisense oligodeoxynucleotide (0.01 to 5 µM) 1 h prior to infection.

3. Infect cultures with HCMV AD169 or HCMV clinical isolate as described in **Subheading 3.3.2.**

4. Remove viral inoculum by aspiration, add DMEM-5% FBS containing an appropriate concentration of fomivirsen, or WC5 (50 µM), or 0.2% DMSO as a control, and incubate in a CO_2 incubator at 37°C for 48 h.p.i..

5. Quantify the HCMV-induced EGFP expression by automated fluorometry assay as described in **Subheading 3.3.4.**

6. Estimate the IE2-dependent EGFP expression at each drug concentration.

7. Determine the 50% inhibitory concentration (IC_{50}) values using dose response curves (*see* **Note 15**).

8. An example of the concentration-dependent inhibitory effect of the IE2-targeting antisense oligodeoxynucleotide fomivirsen on EGFP expression measured in HCMV AD169-infected 2F7 and 1B4 cells at 48 h.p.i., is shown in **Fig. 5** (left panel). In comparison, the same concentrations of fomivirsen were evaluated by a conventional plaque reduction assay in HELFs (**Fig. 5**, right panel). **Figure 6** shows the employment of the EGFP-based cell assay to test the ability of the anti-HCMV 6-aminoquinolone WC5 to inhibit the IE2-dependent transactivating activity *(10).*

4. Notes

1. Small-molecule libraries are either commercially available or can be found at academic facilities. For an example see **Ref. *(2)*.** Compounds can be purchased in lyophilized form and dissolved in DMSO at the desired concentration.

2. Automated dispensers are preferable for maximal standardization of the operations. When the screening is manually performed, a 96-well format is recommended to minimize differences between samples, in particular between the first and last samples dispensed.

3. Always calculate volumes to be aliquoted in excess of what is actually needed for the experiment, particularly when automated operations are carried out.

4. Samples can be either automatically dispensed using a 384-pin array or manually dispensed. When manually dispensed, a 96-well format is recommended in order to avoid variability among first and last samples.

5. If using an automated dispenser, 0.1 µl of each compound at 5 mg/ml is transferred to individual wells using a 384-pin array.

6. To avoid differences among the wells, the use of a multi-channel pipette is recommended.

7. Ensure that the final DMSO concentration in each well is not higher than 1% to avoid non-specific effects of DMSO.

8. Mix equal volume of UL44 and peptides, e.g. 60 µl of UL44 and 60 µl of peptide solution.

9. Prior to incubation with HRP substrate, if there are bubbles in the sample eliminate them by punching with a needle.

10. Alternatively, other commercially available substrates of horseradish peroxidase can be used, such as 3,3,5,5-tetramethylbenzidine (TMB), 5-aminosalicylic acid (5AS), and O-phenylenediamine (OPD).

11. The astrocytoma/glioblastoma cell line U373-MG (ATCC HTB-17) was chosen, as these cells can be routinely infected with HCMV to >90% and are permissive for viral replication *(14)*. The cells were

transfected with plasmids pUL54-EGFP or pUL112/113-EGFP, which contain the UL54 (positions –425/+15) or the UL112/113 (positions –353/+32) viral early gene promoters cloned upstream from the EGFP reporter gene. These viral E gene promoters were chosen on the basis of the negligible basal activity in uninfected cells, strong inducibility upon HCMV infection, and transactivation in response to constitutive IE2 expression. The EGFP plasmid harbours the neomycin-resistance gene that confers drug resistance to transfected cells, so stable transfectants were isolated by G418 selection. One pUL54-EGFP-containing clone designated 2F7 and one pUL112/113-EGFP-containing clone designated 1B4 were selected, on the basis of low background expression levels and a constant increase in EGFP emission upon either HCMV infection or IE2 expression.

12. To minimize the risk of contamination, biosafety level 2 (BSL-2) practices, containment equipment, and facilities are required for all procedures involving HCMV cultivation and manipulation.

13. HCMV infection induces EGFP expression in 2F7 and 1B4 cells in a time- and dose-dependent manner. Under fluorescent microscopy observation, fluorescent cells can be detected as early as 48 h.p.i., with a gradual increase at 72 and 96 h.p.i..

14. Analysis of quantitative EGFP expression by automated fluorometry provides a simple and rapid protocol in which there are only two steps: cell lysis and fluorescence measurement by a plate reader. EGFP quantification by automated fluorometry is sensitive, specific and less expensive than other analytical methods, as it does not require additional extraction procedures, enzymes or immunological reagents. The adaptability of 2F7 and 1B4 cell lines to a 96-wellformat, makes EGFP quantification by automated fluorometry suitable for HTS of large collections of small molecules.

15. Concentrations producing 50% reductions in plaque formation and HCMV-induced EGFP expression (IC_{50}) may be calculated by nonlinear regression using a computer program (e.g., PRISM, version 4.0, GraphPad Software, Inc).

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Figure Legends

Figure 1.Principles of the FP-based protein-protein interaction assay. (**A**) A synthetic peptide that corresponds to the binding region of one of the two protein partners (PROTEIN 1) and is labelled with a fluorophore, is mixed with the other protein partner (PROTEIN 2). The unbound labelled peptide tumbles relatively rapidly; thus, if it is excited with polarized light, by the time emission occurs, the polarization of the emitted light is low (*left panel*). However, upon binding to the interacting partner, the peptide will tumble much more slowly, so the emitted light remains relatively polarized, resulting in an increase in FP (*right panel*). (**B**) Compounds that interfere with the protein-protein interaction will produce a quantifiable reduction in FP.

Figure 2. Typical results of an FP-based assay to detect the interaction between the two subunits, UL54 and UL44, of HCMV DNA polymerase and to search for inhibitors of such interaction. (**A**) Increasing concentrations of wild-type GST-UL44ΔC290 (●), of GST-UL44ΔC290 I135A, a mutant UL44 which does not bind UL54 (▲), of GST (■), or of an unrelated protein (MBP-UL42ΔC340) (▼) were added to 3 nM of a fluorescently labelled peptide corresponding to the C-terminal 22 residues of HCMV UL54 (peptide 1), and FP (as millipolarization units, mP) was measured(reproduced from *(2)* with permission from Elsevier). (**B**) Increasing concentrations of the indicated small-molecule compounds (named AL3, AL5, AL9, AL12, AL18, AL20, and AL21) were added to reaction mixtures containing 2.5 µM GST-UL44ΔC290 and 3 nM labelled peptide 1, and FP (as millipolarization units, mP) was measured (reproduced from *(2)* with permission from Elsevier).

Figure 3. Typical results of an ELISA-based assay to detect the interaction between the two subunits, UL54 and UL44, of HCMV DNA polymerase and to search for inhibitors of such

interaction. (A) Binding of UL44 to UL54 as detected by ELISA. The UL44 protein was added to microtiter wells precoated with 0.2 µg of purified UL54 (●) or uncoated wells (○). Bound UL44 was detected with MAb YL1/2, which was in turn detected with an HRP-conjugated anti-rat antibody (reproduced from *(12)* with permission from American Society for Microbiology). **(B)** The inhibition of the interaction between UL54 and UL44 proteins in the presence of varying concentrations of the indicated small-molecule compounds (named AL3, AL5, AL9, AL12, AL18, AL20, and AL21), and of a synthetic peptide corresponding to the C-terminal 22 residues of HCMV UL54 (peptide 1) as a positive control for UL54-UL44 inhibition was detected by ELISA as described in (A) (reproduced from *(2)* with permission from Elsevier).

Figure 4. Typical results of a fluorescent microscopy assay to detect IE2 driven GFP reporter gene activity during HCMV infection of U373-2F7 and U373-1B4 reporter cells lines. Cells were infected with HCMV AD169 (MOI 5) and the number of EGFP-expressing cells was examined by fluorescence microscopy at multiple times post-infection (shown are 72 and 96 h.p.i.). Representative fluorescence microscopy images at 72 and 96 h.p.i. are shown [modified from *(8)*].

Figure 5. Validation of the EGFP-based reporter cell lines U373-2F7 and U373-1B4 for the assessment of the inhibitory activity of fomivirsen. U373-2F7 and U373-1B4 cells were infected with HCMV AD169 (MOI 5) or mock-infected. Where indicated, the cells were treated with different concentrations of fomivirsen (0.01 to 5 uM) (ISIS 2922) prior to and during infection. At 48 h.p.i., the cells were lysed and assayed for EGFP expression by quantitative automated fluorometry (*left panel*). In comparison, a conventional plaque reduction assay was performed in HELF cell (*right panel*). The calculated IC_{50} of fomivirsen in fluorescence-based experiments was 0.26 µM in 2F7 cells and 0.17 µM in 1B4 cells. The calculated IC_{50} of fomivirsen in traditional plaque assays was 0.16 µM in HELFs.

Taken together, these studies indicate that the sensitivity of the EGFP-based fluorescence assay is similar to that of the plaque reduction assays in measuring the antiviral activity of fomivirsen *(8)*.

Figure 6. Investigation of the ability of the 6-aminoquinolone WC5 to inhibit the IE2-dependent transactivation of HCMV viral gene promoters by using the EGFP-expressing U373-2F7 and U373-1B4 reporter cells. 2F7 and 1B4 cells were mock-infected or infected with HCMV AD169 (MOI 0.1) and then treated with WC5 (50 µM), fomivirsen (5 µM) or DMSO as a control. At 48 h.p.i., the cells were examined by confocal fluorescence microscopy to evaluate the number of EGFP-expressing cells [reproduced from *(10)* with permission from American Society for Microbiology].

Figure 1

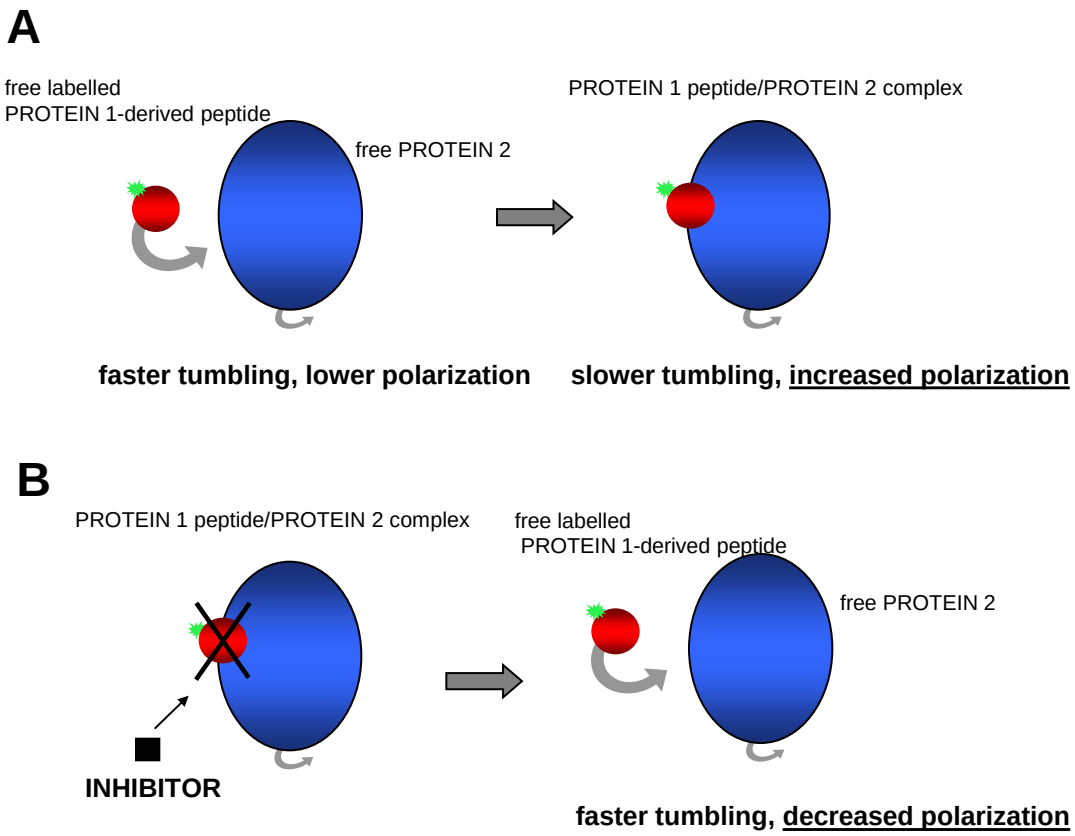

A
free labelled
PROTEIN 1-derived peptide
free PROTEIN 2
PROTEIN 1 peptide/PROTEIN 2 complex
faster tumbling, lower polarization
slower tumbling, increased polarization
B
PROTEIN 1 peptide/PROTEIN 2 complex
free labelled
PROTEIN 1-derived peptide
free PROTEIN 2
INHIBITOR
faster tumbling, decreased polarization

Figure 2

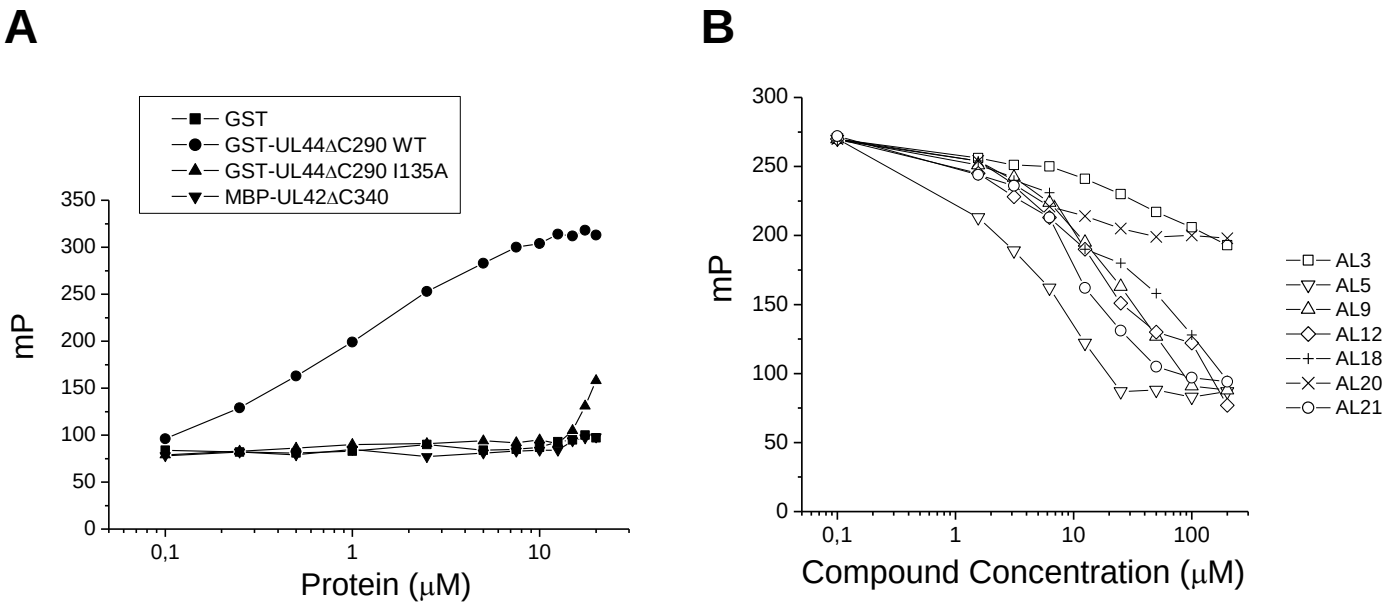

A
GST
GST-UL44ΔC290 WT
GST-UL44ΔC290 I135A
MBP-UL42ΔC340
mP
Protein (μM)
B
mP
Compound Concentration (μM)
AL3
AL5
AL9
AL12
AL18
AL20
AL21

Figure 3

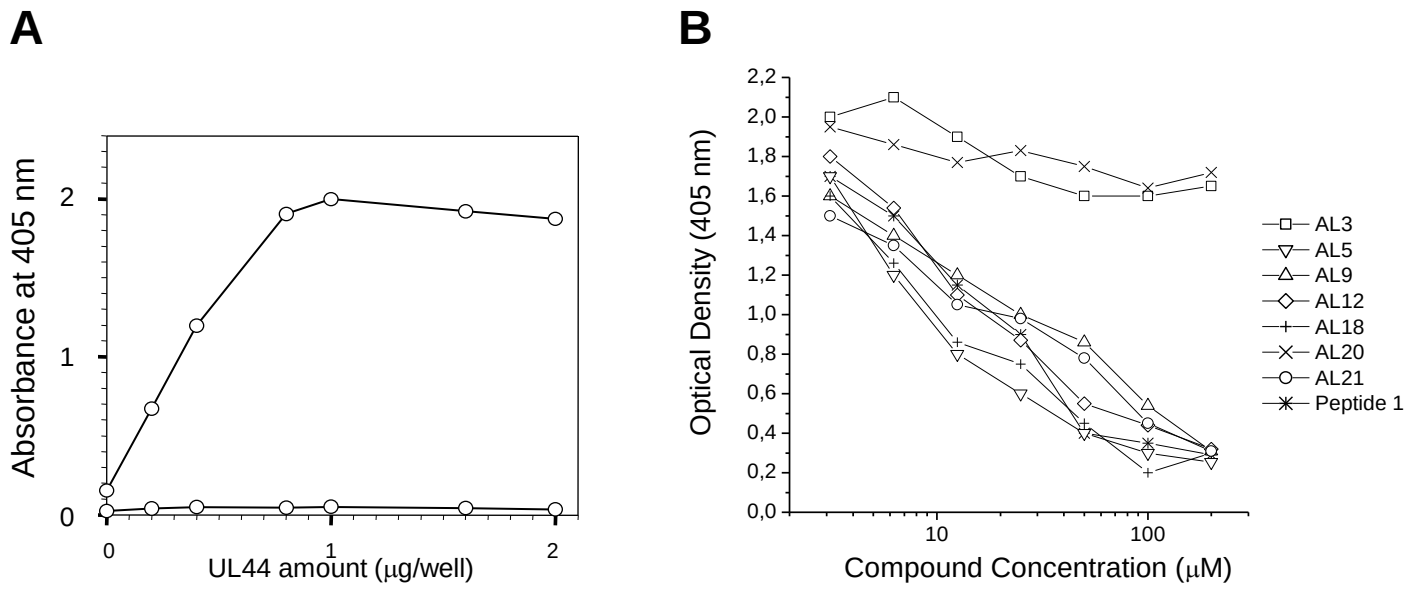

A
Absorbance at 405 nm
0
1
2
0
1
2
UL44 amount (μg/well)
B
Optical Density (405 nm)
2,2
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
10
100
Compound Concentration (μM)
AL3
AL5
AL9
AL12
AL18
AL20
AL21
Peptide 1

Figure 4

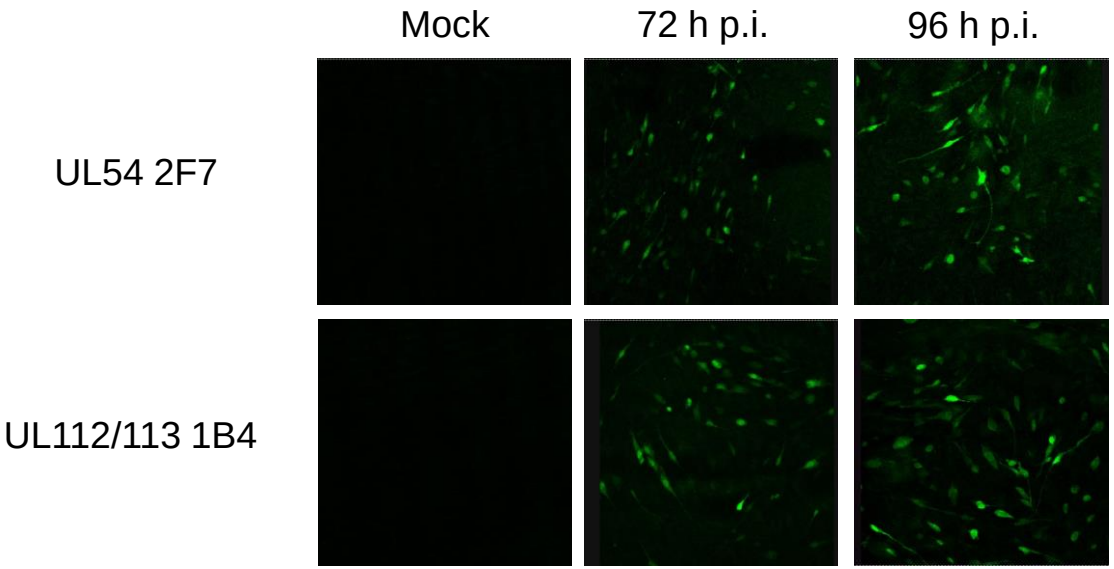


Figure 5

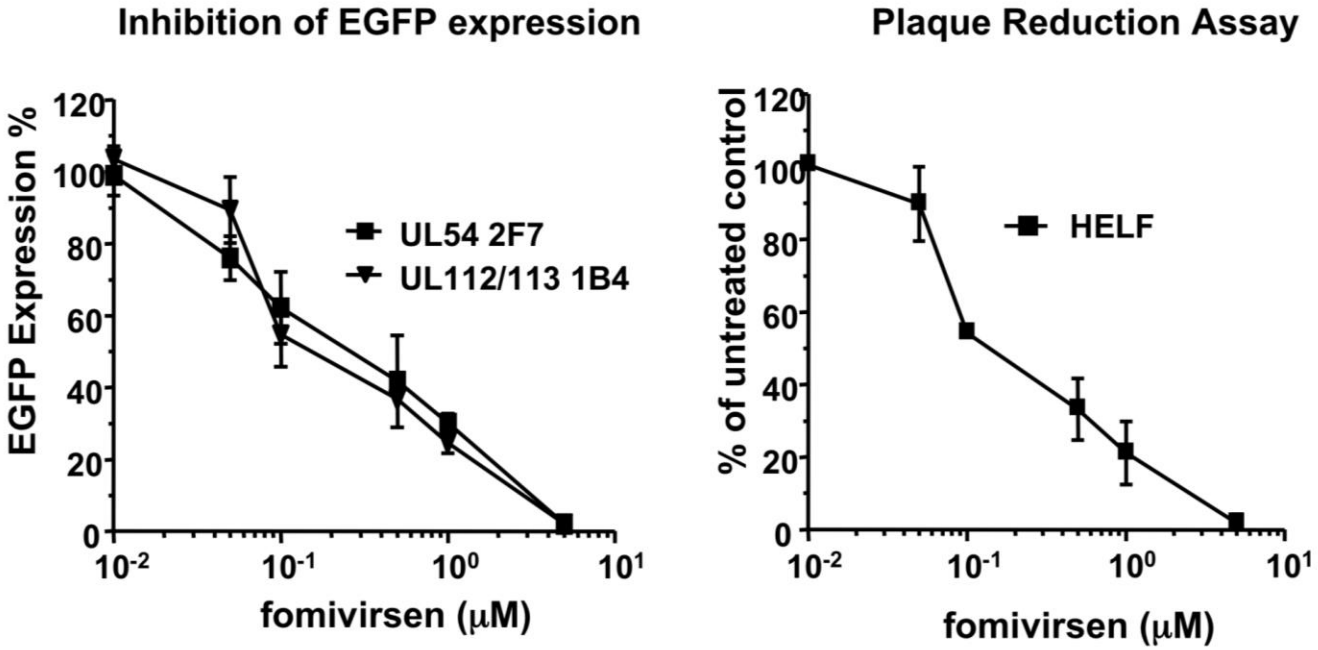
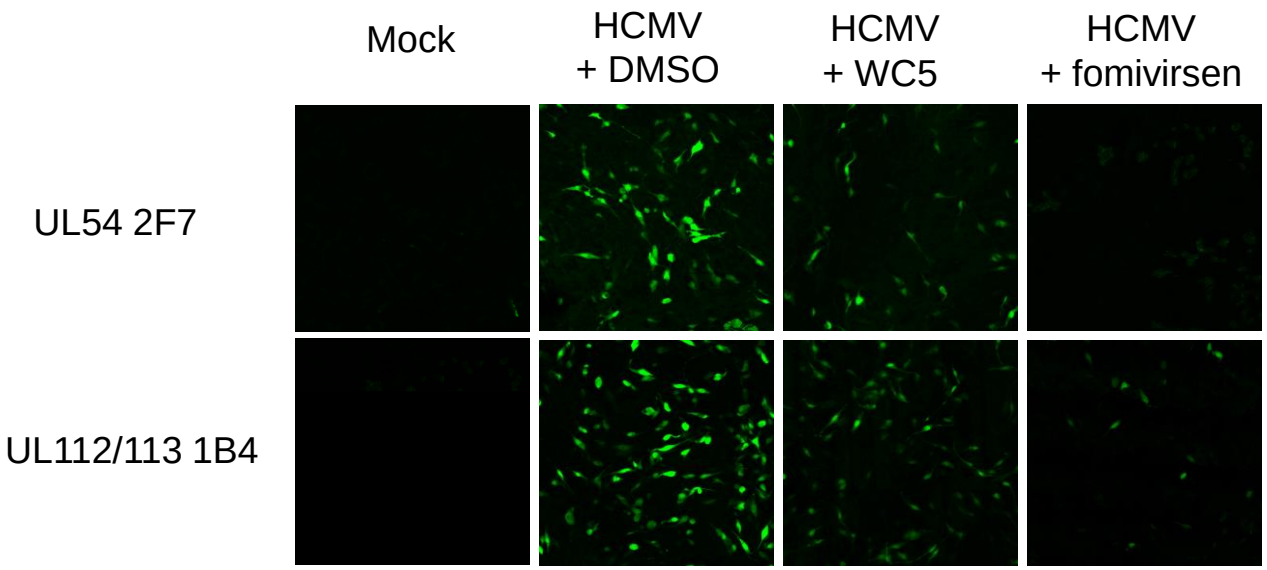

Inhibition of EGFP expression
EGFP Expression %
120
100
80
60
40
20
0
UL54 2F7
UL112/113 1B4
10-2
10-1
100
101
fomivirsen (μM)
Plaque Reduction Assay
% of untreated control
120
100
80
60
40
20
0
HELF
10-2
10-1
100
101
fomivirsen (μM)

Figure 6


Mock
HCMV
+ DMSO
HCMV
+ WC5
HCMV
+ fomivirsen
UL54 2F7
UL112/113 1B4